AD_____

AWARD NUMBER: W81XWH-04-1-0501

TITLE: Radiation Sensitization in Breast Cancer via Targeting Survivin Expression

PRINCIPAL INVESTIGATOR: Bo Lu, M.D., Ph.D.

CONTRACTING ORGANIZATION: The Vanderbilt University
Nashville, Tennessee 37235-7745

REPORT DATE: May 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-05-2006	Annual	20 Apr 2005 – 19 Apr 2006

4. TITLE AND SUBTITLE

Radiation Sensitization in Breast Cancer via Targeting Survivin Expression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0501

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Bo Lu, M.D., Ph.D.

E-Mail: bo.lu@vanderbilt.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Vanderbilt University
Nashville, Tennessee 37235-7745

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We proposed to determine whether overexpression of survivin results in radioresistance and the possible mechanisms; whether regulators of survivin serve as targets for radiosensitization. We also found that deregulation of survivin in breast cancer is mediated by Stat3 (Signal transducer and activator of transcription). Co-inhibition of survivin and Stat3 results in significantly increased sensitization of breast cancer. These concepts are being tested in animal models of breast cancer.

15. SUBJECT TERMS
Breast cancer, radiotherapy, surviving, CDK

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	10	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents:

1. Introduction:

We have proposed to determine whether overexpression of survivin results in radioresistance and the possible effects on cell death mechanisms.

2. Body:

To accomplish the proposed studies: we have the following Statement of Work for the first 12 months:

Task 1. To determine whether overexpression of survivin results in radioresistance and the possible mechanisms (Months 1-9):

We have shown survivin overexpression leads to radiation resistance as demonstrated in reference 1.

Task 2. To determine whether irradiation downregulates p34CDC2 and its mechanism in vascular endothelium and whether CDK inhibitors sensitize it to radiation injury. (Months 9-19):

Our preliminary data showed that inhibitors of survivin and CDK result in synergistic radiosensitization.

Task 3. To determine the mechanism of survivin deregulation in breast cancer and whether inhibition of survivin or its regulator, p34CDC2 abolishes radioresistance. (Months 20-28):

We found that deregulation of survivin in breast cancer is mediated by Stat3. We also found that mTOR inhibitors radiosensitize breast cancer via induction of autophagy. Co-inhibition of survivin and its partner, aurora B induced mitotic arrests and radiosensitization.

Task 4. To determine the biological significance of combining survivin inhibitors or CDK inhibitors with radiotherapy in xenograft models of breast cancer (Months 29-36)

These experiments are ongoing.

Results Obtained: Please see the attached results.

3. Key Research Accomplishments:

1. Survivin overexpression leads to radioresistance.
2. Inhibitors of survivin and CDK result in radiosensitization.
3. Stat3 mediates deregulation of survivin in breast cancer.
4. mTOR inhibitors radiosensitize breast cancer via induction of autophagy.
5. Co-inhibition of survivin and aurora B induces mitotic arrests and radiosensitization.

4. Reportable outcomes:

1. Kwang Woon Kim and Bo Lu. Stat3 mediates transcriptional downregulation of survivin following irradiation. (accepted by Molecular Cancer Therapeutics, 2006).

Abstract: Stat3 and Survivin are constitutively upregulated in various human tumor cells. We previously found Survivin to be significantly reduced in response to radiation in human umbilical vein endothelial cells (HUVECs), but not in tumor cell lines. In this study, we examined the effect of Stat3 on Survivin expression in irradiated HUVECs and breast cancer cells. In HUVECs, we manipulated Stat3 levels using various adenovirus vectors overexpressing active or dominant-negative Stat3. Stat3 activation and Survivin levels were examined by western blotting. Clonogenic and endothelial morphogenesis assays were used to

determine cell survival and angiogenesis following irradiation and inhibition of either Stat3 or Survivin. Survivin was significantly increased by over-expression of an active Stat3 (Stat3-C). Following irradiation, the level of phosphor-Stat3 Tyr-705 but not phospho-Stat3 Ser-727 was reduced in HUVECs whereas it remained unchanged in irradiated breast cancer cells. Correspondingly, Stat3 DNA-binding activity following irradiation was specifically down-regulated in HUVECs but not in breast cancer cells. Mutation of Tyr-705 abolished radiation-induced downregulation of Survivin. Clonogenic and endothelial cell morphogenesis assays suggested that DN-Stat3 and DN-Survivin together resulted in the greatest radiosensitization of MDA-MB-231, decreasing angiogenesis and cell survival. In conclusion, Stat3 modulates Survivin and both are potential therapeutic targets for radiation sensitization in breast cancer.

I). Regulation of Survivin by STAT3.

To determine whether STAT3 regulates the expression of survivin in HUVEC cells, we transduced HUVECs with adenovirus vector (pAdCMVpLpA(-)loxp-SSP) or adenovirus overexpressing antisense STAT3-C as a negative control. Adenoviruses overexpressing constitutively active STAT3-C, or DN STAT3 (Y705F) were used to increase or to decrease Stat3 activity, respectively. Fig. 1 shows the autoradiograph of Western immunoblots. Survivin protein level was markedly elevated in the cells following overexpression of STAT3-C. However, survivin was not induced in the cells infected with negative controls. DN STAT 3(Y705F) reduced the level of phospho-Stat3 Tyr 705 as well as the expression of survivin.

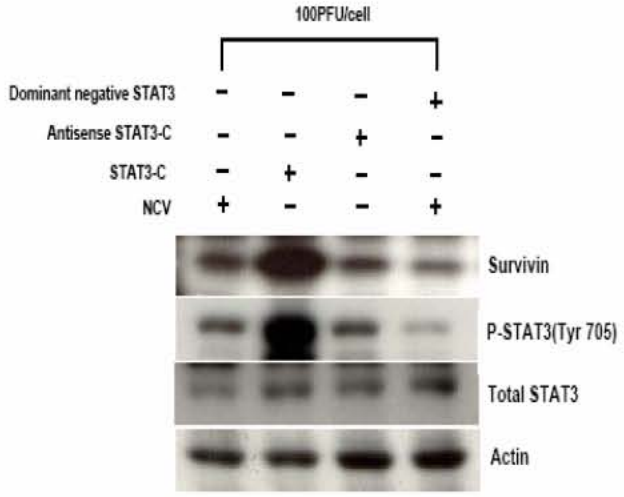


Figure 1. STAT3 regulated survivin protein in HUVECs. HUVECs were transduced with adenoviral vector control (NCV), adenoviruses overexpressing STAT3C, antisense STAT3C or DN STAT3 (Y705F). After 24h of infection, the cells were harvested and fifty microgram total proteins per lane were loaded on 15% SDS-PAGE and subjected to Western blot analysis using various antibodies. Actin was probed to demonstrate equal loading.

II). Irradiation attenuates the active form of phospho-Tyr 705 STAT3.

To examine whether irradiation affects the activation of STAT3, HUVEC, MDA-MB-231 and MCF-7 breast cancer cells were irradiated with 3Gy and harvested at 0, 30, 60, and 240min. Western blot analysis was performed using antibodies against phospho-Tyr 705 STAT3, phospho-Ser 727 STAT3 and total STAT3. As shown in Fig. 2A, phospho-Tyr 705 STAT3 was reduced at 30min and slowly recovered at 240min in HUVECs. However, irradiation did not change the level of phospho-Ser 727 STAT3 in HUVECs. (Fig 2B) To determine whether irradiation affects STAT3 activation by growth factors, we examined the effect of irradiation on STAT3 phosphorylation following pre-treatment of HUVEC cells with epidermal growth factor (EGF). Since STAT3 is activated by EGF signaling (22), HUVEC cells were pre-treated with EGF prior to be subject to either 0 or 3Gy. Stimulation with EGF resulted in an increased level of phospho-Tyr 705 STAT3 whereas irradiation following the EGF treatment reduced the phosphorylation of Tyr 705 STAT3. However, phospho-Ser 727 STAT3 levels showed no change following either EGF or irradiation (data not shown).

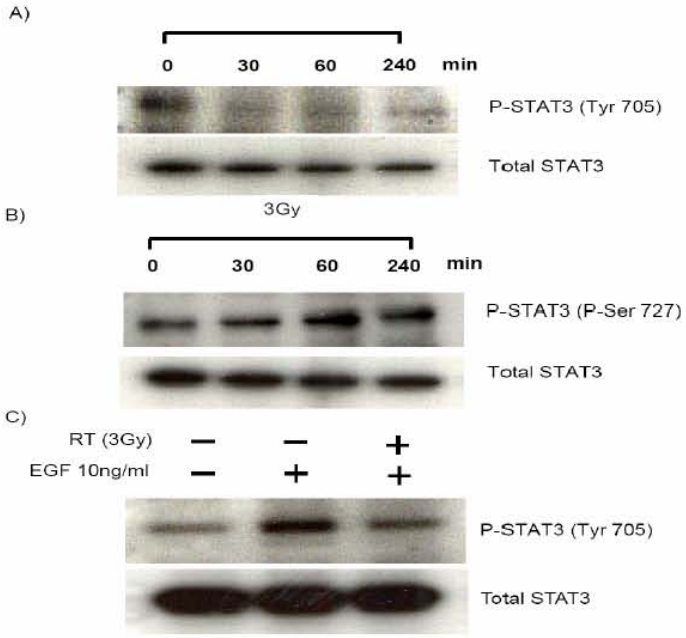
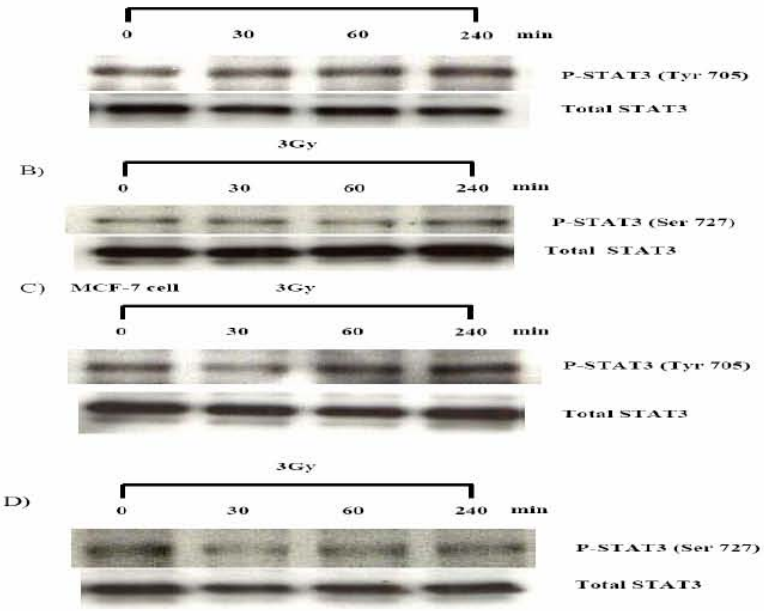


Figure 2. Radiation reduced phospho-Tyr 705 STAT3, not phospho-Ser 727 STAT3 in HUVECs.
HUVECs cells were irradiated with 3Gy. Fifty microgram total proteins per lane were immunoblotted for p-STAT3. (Tyr 705 and Ser 727) and total STAT3. A: p-STAT3 (Tyr705), B: p-STAT3 (Ser 727) C: Radiation interfered with EGF-induced tyrosine phosphorylation (Tyr 705) of STAT3 in the HUVECs.

In the breast cancer cells, both phospho-Tyr 705 STAT3 and phospho-Ser 727 STAT3 levels were not significantly altered by irradiation. (Fig.3)

Figure3. Radiation does not affect STAT3 in breast cancer cells. MDA-MB-231 and MCF-7 breast cancer cells were irradiated with 3Gy. Total cell lysates were extracted at indicated time points. Fifty microgram total proteins per lane were immunoblotted for p-STAT3 (Tyr 705 and Ser 727) and total STAT3. A: MDA-MB-231, p-STAT3 (Tyr705) B: MDA-MB-231, p-STAT3 (Ser 727) C: MCF-7, p-STAT3 (Tyr705) D: MCF-7, p-STAT3 (Ser727).



III). Radiation reduced DNA binding activity of STAT3 in HUVECs:

To determine whether dephosphorylation of STAT3 affects DNA-binding activity, we irradiated HUVECs with 3 Gy. STAT3 DNA binding activity was determined via electrophoretic mobility shift assay (EMSA) using 10ug nuclear protein and a 32-P-labeled oligonucleotide probe containing a consensus-binding motif for STAT3. As shown in Fig. 4, STAT3 activity was reduced at 30min and had slight recovery at 240 min after irradiation. Consistent with Western blot analysis of the attenuated phospho-Tyr 705 STAT3 (Fig.2A), this result also suggests that irradiation inhibited DNA binding activity of STAT3 in HUVEC cells.

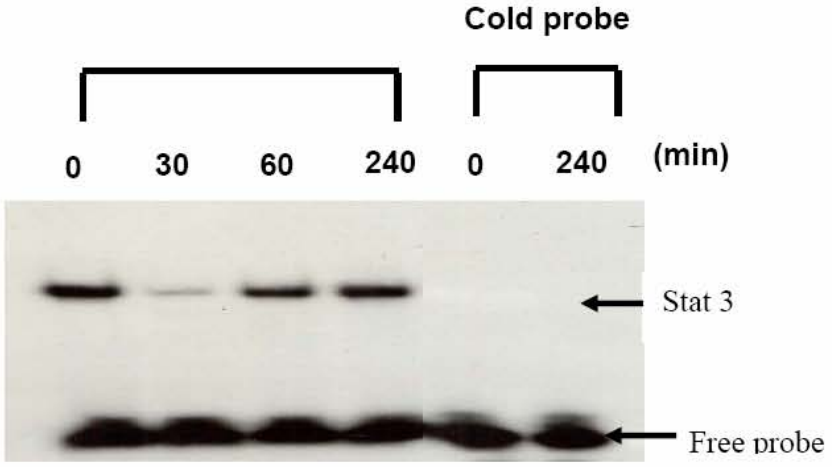


Figure 4. Radiation reduced DNA binding activity of STAT3 in HUVECs.
HUVEC cells were irradiated with 3Gy. Nuclear extracts were prepared at indicated time points. 5ug of nuclear extracts were used for mobility shift assay. Competitor assay was also performed using specific cold probe.

IV). Mutation of Tyr 705 in STAT3 abolished radiation-induced downregulation of survivin:

To determine whether dephosphorylation of STAT3 Tyr 705 is essential for radiation-induced downregulation of survivin, we obtained human mammary epithelial cells overexpressing either wild type STAT3 or Y705F STAT3 mutant from our collaborator. As shown in Figure 5, survivin protein level decreases in wild type cells whereas it remained unchanged in mutant cells following irradiation. This result suggests that Tyr 705 of STAT3 is essential for radiation-mediated downregulation of survivin.

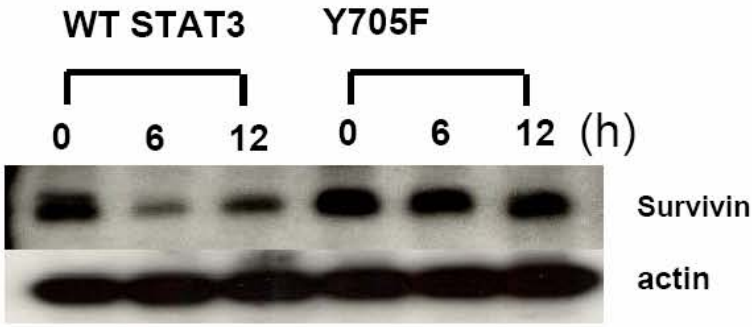
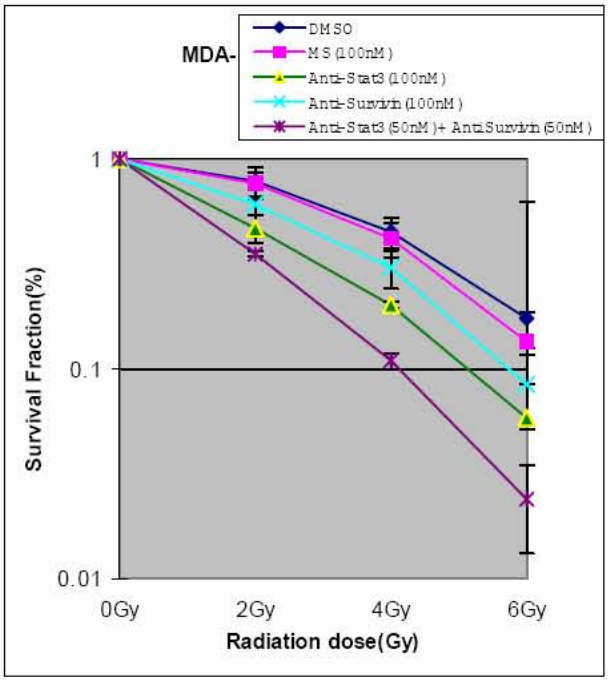


Figure 5. Tyr 705 of STAT3 is essential for radiation-induced downregulation of survivin.
HME1 cells stably transfected with WT STAT3 or Y705 STAT3 expression plasmids were treated with 3Gy. Protein extracts were collected at 0, 6 and 12hr following irradiation. Survivin and beta-actin were probed by western analyses.

V). Combined inhibition of STAT3 and survivin increases radiation sensitization in MDA-MB-231 and MCF-7 breast cancer cells.

To determine whether combined inhibition of both STAT3 and survivin sensitizes MDA-MB-231 and MCF-7 breast cancer cells to radiotherapy, clonogenic assays were performed and are shown in Figure 6. As shown, cells transfected with ASO against STAT3 or ASO against survivin had less surviving colonies as compared to the cells transfected by the control oligo (MS). Also, inhibition of STAT3 resulted in less survival than inhibition of survivin. Furthermore, STAT3 ASO combined with survivin ASO showed the greatest radiosensitization of both MDA-MB-231 and MCF-7 breast cancer cells, although STAT3 ASO alone also showed strong radiosensitizing activity. Therefore, these results suggest that STAT3 and survivin could be important targets for enhancing radiosensitization in breast cancer cells.

A). MDA-MB-231



B). MCF-7

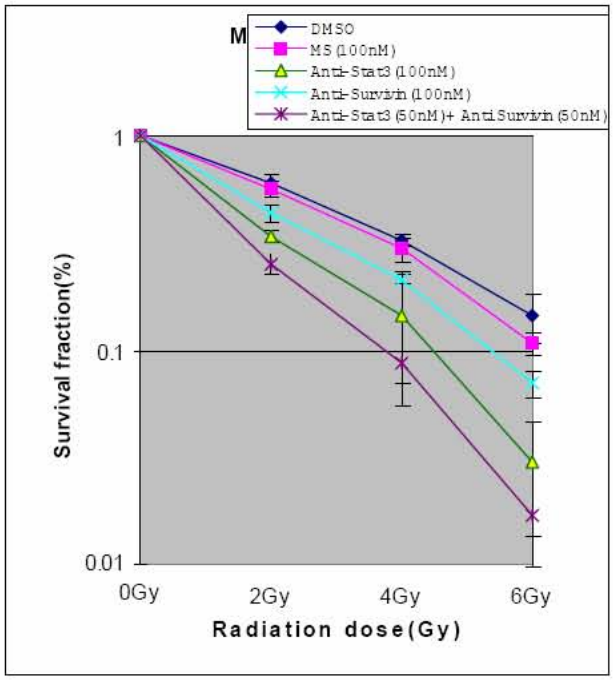


Figure 6. Combined inhibition of STAT3 and survivin increases radiation sensitization in breast cancer cells

MDA-MB-231 and MCF-7 breast cancer cells were transfected with either nothing or MS control oligo (100mM) or ASO survivin (100mM), ASO STAT3 (100mM) ASO STAT3 (50mM) mixed with ASO survivin (50mM). They were then treated with 0, 2, 4 or 6 Gy. After 10 days colonies were stained and scored. Data is shown as the mean +/- SD.

2. Jeffrey M. Albert, Kwang Woon Kim, Carolyn Cao, and **Bo Lu.** Targeting the Akt/mTOR pathway for radiosensitization of breast cancer. Molecular Cancer Therapeutics **May 1, 2006; 5 (5)**.):1183-9 (published).

Abstract: The PI3K/Akt pathway is known to be activated by radiation. The mammalian target of rapamycin (mTOR) is downstream of Akt, and we investigated the effects of radiation on Akt/mTOR signaling in breast cancer cell models. RAD001 (everolimus), a potent derivative of the mTOR inhibitor rapamycin, was used to study the effects of mTOR inhibition, as the role of mTOR inhibition in enhancing radiation remains unexplored. RAD001 decreased clonogenic cell survival in both breast cancer cell lines MDA-MB-231 and MCF-7, though the effect is greater in MDA-MB-231 cells. Irradiation induced Akt and mTOR signaling, and this signaling is attenuated by RAD001. The radiation-induced signaling activation is mediated by PI3K, since inhibition of PI3K with LY294002 inhibited the increase in downstream mTOR signaling. Additionally, caspase-dependent apoptosis is an important mechanism of cell death when RAD001 is combined with 3 Gy radiation, as shown by induction of caspase 3 cleavage. An increase in G2/M cell cycle arrest was seen in the combination treatment group when compared to controls, suggesting that cell cycle arrest may have been a contributing factor in the increased radiosensitization seen in this study. We conclude that RAD001 attenuates radiation-induced pro-survival Akt/mTOR signaling and enhances the cytotoxic effects of radiation in breast cancer cell models, showing promise as a method of radiosensitization of breast cancer.

5. Conclusions: We have found that deregulation of survivin in breast cancer is mediated by Stat3. This could lead to radioresistance. Inhibitors of survivin, Stat3, and mTOR enhance therapeutic effects of radiation. We will investigate biological consequences of these inhibitors in animal models.

6. Recent Publications in the open literature that acknowledged this funding:

1). Kwang Woon Kim and **Bo Lu.** Stat3 mediates transcriptional downregulation of survivin following irradiation. (accepted by Molecular Cancer Therapeutics, 2006).

2). Jeffrey M. Albert, Kwang Woon Kim, Carolyn Cao, and **Bo Lu.** Targeting the Akt/mammalian target of rapamycin pathway for radiosensitization of breast cancer. Mol Cancer Ther 2006 5: 1183-1189.

3). Carolyn Cao, Eric T. Shinohara, Ty K. Subhawong, Ling Geng, Kwang Woon Kim, Jeffrey M. Albert, Dennis E. Hallahan, and **Bo Lu** Radiosensitization of lung cancer by nutlin, an inhibitor of murine double minute 2 Mol Cancer Ther 2006 5: 411-417